6. On the Role of Lipemia Clearing Factor in Lipid Transport

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THE STATE OF LIPIDS in plasma and tissues has been the subject of considerable study during recent years.[1] These studies have shown that lipids exist virtually exclusively in the form of lipoprotein complexes. Our understanding of the phenomenon of lipid transport and metabolism must, therefore, be directed, at least in part, toward an examination of the enzymatic and hormonal control of lipoprotein synthesis and transformation. In addition, it is clear that the study of aberrations in lipid metabolism occurring in various metabolic diseases must similarly involve such considerations.

The chemistry and physical description of the various lipoproteins of plasma in health and disease has been reviewed extensively of late (1-8). I will not, therefore, attempt to cover this comparative material in detail at the present time, particularly as regards specific disease entities. Instead, I would like to concentrate on the more general aspects of lipoprotein metabolism in plasma.

Lipoproteins form a class of compounds with varying densities and containing different proportions of protein, phospholipid, cholesterol, and triglycerides. These proteins

[1] The various original studies from the National Heart Institute described in connection with the following discussion were carried out by Drs. E. Boyle, J. Bragdon, R. Brown, R. Gordon, R. Havel, E. Korn, and C. Anfinsen.

may be studied conveniently by a variety of techniques, including ultracentrifugal, electrophoretic and chemical methods. Techniques introduced by Gofman and his collaborators (6), Gurd and Oncley (9), and Pedersen (10) permit the separation of the various density classes by means of flotation at high centrifugal speeds under chosen conditions of solvent density. At a density of 1.063, for example, the β lipoprotein class as well as lipoproteins of uncertain composition of even lower density, can be separated from the α lipoprotein components. Similar separations are possible by electrophoresis on starch (8) or by ethanol fractionation (7).

In the majority of diseases involving abnormal lipid metabolism, the β lipoprotein fraction of plasma is considerably increased in concentration, whereas the α lipoproteins appear to decrease. The correlations earlier observed between the levels of plasma cholesterol and the incidence of atherosclerosis or of such diseases as diabetes and myxedema are now interpretable on the basis of the fact that the low density lipoproteins constitute the major vehicle for cholesterol transport in plasma. It would appear a fruitful approach to the study of such diseases, therefore, to examine the mechanisms by which the normal pattern of plasma lipoproteins is maintained.

Immunological studies have indicated that the normal β_1 lipoprotein is strongly cross-antigenic with lipoproteins of lower density and higher S_f * (11). In addition, it has been frequently demonstrated that these latter molecules are rapidly degraded in the normal individual, following ingestion of fat (4), in the direction of the normal β lipoprotein component, passing successively through stages of higher

* The symbol S_f indicates Svedberg units of flotation under the conditions described by Gofman et al. (6).

density and lower triglyceride content during their metabolism. One explanation for this progressive change is the possibility that the very low-density lipoproteins may simply constitute normal β_1 lipoprotein molecules containing varying amounts of triglyceride in their basic matrix.

Heparin induced clearing factor. During the past two years our group has concerned itself with the study of an enzymatic mechanism stimulated by heparin administration which may play an important part in the normal degradation of the transient low-density lipoproteins. In 1943, Hahn (12) observed that the injection of heparin caused the rapid disappearance of chylomicrons from the post-absorptive plasma of dogs. These studies have been greatly extended in numerous laboratories (13–21) and the references quoted at the end of this discussion list many of the observations that have been made since Hahn's original discovery.

When heparin is injected intravenously into humans or experimental animals whose plasmas contain elevated levels of chylomicrons or of abnormal low-density β lipoproteins, these components rapidly disappear from the plasma. It has also been possible to produce the clearing factor responsible for these changes *in vitro* as well as *in vivo* (16, 22). Thus, by the incubation of normal plasma, containing no detectable clearing factor activity, with heparin in the presence of extracts from various tissues, one can demonstrate the production of triglyceride splitting activity in the incubation mixture. The tissue factor which brings about this heparin dependent production of clearing factor appears to be most active in lung, heart and pyloric mucosa. Tissue factor has not been solubilized as yet but appears to be localized in the submicroscopic particles of the various tissues studied.

Clearing factor may be assayed by measuring its ability to cause a decrease in the turbidity of alimentary lipemic sera, turbid sera from alloxan diabetic rabbits or synthetic triglyceride emulsions. We have more recently assayed its enzymatic activity by measuring the release of fatty acids and/or glycerol during triglyceride hydrolysis, thus obviating many of the difficulties involved in the measurement of turbidity changes in relatively unstable emulsions (23). In Table 1 are presented the results of an experiment in which

TABLE 1

CHANGES IN FREE FATTY ACID AND GLYCEROL (HCHO RELEASED BY PERIODATE) CONTENT, AND IN TURBIDITY DURING THE CLEARING OF COCOANUT OIL EMULSION.

Time (min.)	Turbidity (E_{500})	% of max. change	Fatty Acid μM	% of max. change	Glycerol μM	% of max. change
0	0.578	—	4.2	—	0	—
15	0.468	23	8.2	29	0.7	17
30	0.306	56	10.6	46	1.1	26
60	0.194	79	14.9	77	1.7	41
120	0.108	97	16.7	90	2.9	69
180	0.094	100	18.2	100	4.2	100

(Each reaction tube contained 2 ml. cf (plasma), 3 ml. 5% bovine albumin, 4 ml. buffer (30), and 1 ml. 0.5% cocoanut oil emulsion. Incubated at 37° C. One tube analyzed at each time indicated.)

fatty acid and glycerol production was followed during the clearing of a cocoanut oil emulsion. The clearing factor employed in this experiment was an alcohol fraction of plasma from which serum albumin had been completely removed by washing the insoluble alcohol precipitate exhaustively with water. Two major observations can be made from this experiment. First, it is clear that, although fatty acid production follows a more or less parallel course with turbidity change, glycerol production lags considerably behind. This finding indicates the intermediary accumulation of di- and monoglycerides in a nonturbid, solubilized form.

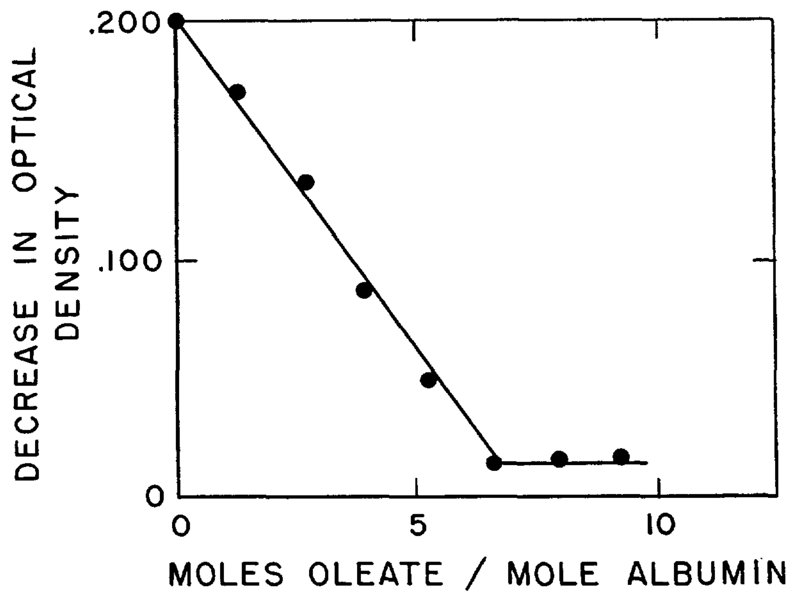

Figure 1. Inhibition of lipemia clearing reaction by oleate.

Further, the data require the postulation of a solubilizing component for these intermediary products.

It will also be noted that the reaction mixture contained added serum albumin, which appears to be an obligatory component of the clearing system. Thus, in these partially purified clearing factor preparations, one can demonstrate that free binding sites on serum albumin, specific for fatty acids, must be available for the normal functioning of the clearing reaction (21). In Figure 1, for example, is shown the results of an experiment in which a purified clearing factor preparation was allowed to attack a cocoanut oil emulsion in the presence of albumin previously treated with increasing quantities of oleate ions. The data indicate that the hydrolysis of triglycerides is essentially completely inhibited when approximately 7 to 8 moles of fatty acid are bound per mole of albumin.

It is possible to demonstrate a further requirement of the system with certain purified preparations of the enzyme. Table 2, for example, shows the marked stimulatory effect

TABLE 2

EFFECT OF ALBUMIN AND SERUM ON PURIFIED CLEARING FACTOR.

Cofactor supplied to purified clearing factor	Decrease in optical density after 1 hr.	2 hr.
0	.004	.009
.2 cc. 5% albumin	.046	.069
.2 cc. 5% albumin + .005 cc. normal serum	.109	.159

of a small amount of normal serum on the catalytic activity of clearing factor in the presence of saturating quantities of serum albumin. The nature of this so-called "coprotein" is, at present, unknown. It is both heat labile and non-dialyzable and therefore presumably protein in nature. The possibility that it is involved in the di- and monoglyceride solubilizing effect mentioned earlier is under investigation.

The preliminary chemical and physical observations that have been made permit us to write a generalized equation for clearing factor action as shown in the equation below:

$$\begin{array}{l}\text{Low density} \\ \text{lipoproteins} + \text{albumin} \xrightarrow[\text{(" coprotein ")}]{\text{clearing factor}} \\ \text{higher density} \\ \text{lipoproteins} + \text{albumin (fatty acid)}_n + \text{glycerol} \\ \qquad (+ \text{" solubilized " mono- \& diglycerides}) \end{array} \tag{1}$$

Evidence for the normal occurrence of the clearing reaction. Experiments such as those described above indicate that an enzymatic component can be isolated from plasma following heparin injection which has the ability to hydrolyze triglycerides and to mimic the changes which occur normally in plasma after fat ingestion. It has been of particular interest to us to determine whether or not this system plays a normal physiological role in lipid transport.

The requirement for heparin in the production of clearing factor both *in vivo* and *in vitro* has suggested, as one possibility, that heparin may be present as a component prosthetic group of clearing factor. As one means of attacking this problem, we have undertaken the purification of clearing factor to permit direct chemical testing of these preparations for heparin. The experiment in the next table illustrates a method which we feel will yield the purification desired (Table 3). Purification is based on the fact that clearing factor forms a complex with triglyceride emulsions permitting the centripetal separation of the complexed enzyme from other plasma components. As indicated, extensive purification can be obtained in a single step. At present, however, it has not been possible to apply this technique on a large enough scale to obtain sufficient quantities of the enzyme for detailed study.

An inherent difficulty of this method has been its unreproducibility when applied to different plasma samples. This may have been due in part to the competition for clearing factor we have observed between the lipids of low density lipoproteins and of triglyceride emulsions (30).

TABLE 3

PROTOCOL OF A TYPICAL PURIFICATION EXPERIMENT ON CLEARING FACTOR.

8 ml. plasma + 8 ml. 1% cocoanut oil emulsion. Layered under saline in 40 rotor-tube. Centrifuged at 30,000 r.p.m., ½ hr., 5° C. Oil layer resuspended, mixed with starch granules, lyophilized, and oil removed with hexane at –20° C. Enzyme eluted with buffer (equiv. to original plasma volume).

ASSAYS

	$\Delta\epsilon/30$ min/cc	ϵ_{280} (undiluted)	Act/ϵ_{280}	Fold Purif.
Orig. Plasma	0.90	78.6	0.011	—
Eluted CF	0.43	0.36	1.2	108x

TABLE 4

PRELIMINARY STEPS IN THE PURIFICATION OF CLEARING FACTOR FROM DOG PLASMA.

	Volume (ml.)	Units/ml	Mg/ml	Specific Activity	Total * units
Plasma	100	0.63	70	0.9	63
Euglob. fraction	150	0.88	11	8.0	132
5×10^{-4} M. Ba^{++} supernatant	300	0.32	1.1	29.0	96

* The figures in this column suggest the removal of an inhibitory substance during purification.

The complexing procedure is now being applied to preparations on which an effort has been made to remove the variable β lipoprotein components. Table 4 shows the preliminary treatment now employed on active plasma samples before the addition of the complexing emulsion. Step 1 in this procedure is essentially the euglobulin precipitation method of Nikkilä (3). The activity in the supernatant

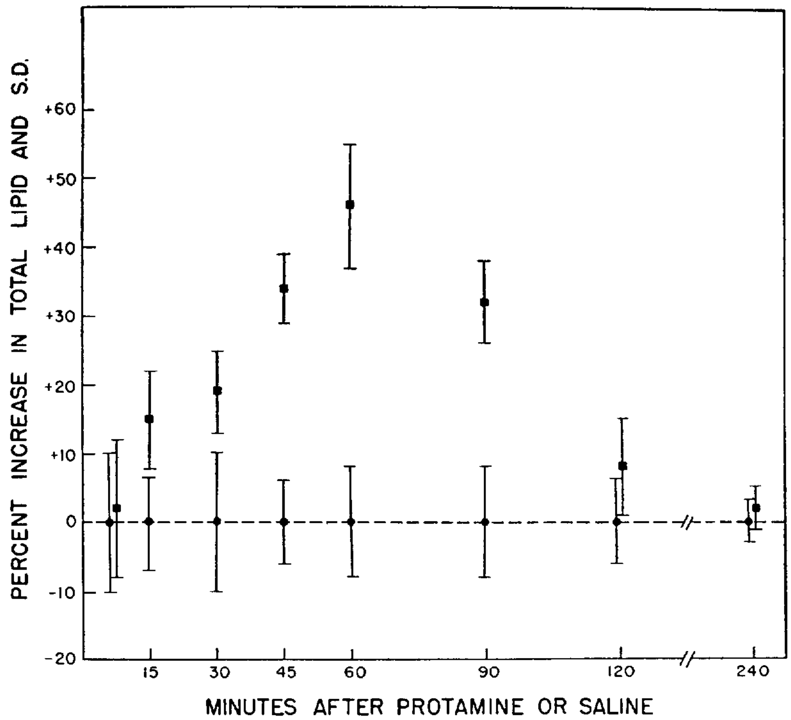

Figure 2. The effect of protamine administration to fasting rats on the level of total plasma lipids.

resulting from barium ion treatment is generally 25–30 fold concentrated over plasma and the yield is essentially quantitative.

Various pieces of indirect evidence provide inferential support for the presence of heparin in clearing factor. Several investigators (25–27) have demonstrated that the injection of the anti-heparin agent, protamine, can reverse the clearing effect of heparin in alimentary lipemia. More recently, Drs. Bragdon and Havel (28) have shown that the administration of small amounts of protamine to fasting rats results in a transient increase in the level of plasma lipids with a specific rise in the content of β lipoproteins of the density class S_f 10 and above (Fig. 2). Heparin administration

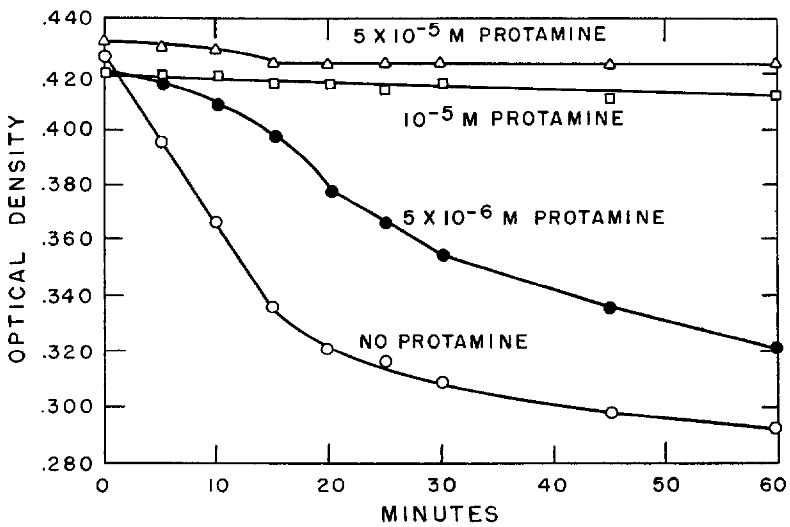

Figure 3. The inhibitory effect of protamine on crude clearing factor activity.

reverses this effect of protamine. These results support the hypothesis that very low levels of clearing factor are present in normal plasma, which are detectable only with difficulty and after considerable concentration (3) by direct methods.

The presence of heparin in clearing factor is also suggested by the inhibitory effect of protamine on crude or partially purified clearing factor preparations. The data in Figure 3 indicate that protamine at low levels can completely inhibit enzymatic activity as measured, in this instance, by the turbidity method on chylomicrons prepared from the serum of alloxan-diabetic rabbits. This inhibitory effect is also shown by the determination of glycerol production as

TABLE 5

EFFECT OF HIGH IONIC STRENGTH AND PROTAMINE ON CLEARING FACTOR AND LIPASE ACTIVITY.

Tube No.		μM Glycerol produced/30 min
1	cf control	0.187
2	+ 10^{-5} M protamine	0.090
3	+ 1 M NaCl	0
4	lipase control	0.397
5	+ 10^{-5} M protamine	0.384
6	+ 1 M NaCl	0.417

Clearing factor (cf—15 mg. alcohol fraction (30) per ml. 0.125 M NH_3–NH_4Cl buffer, pH 8.5 preincubated with salt and protamine at levels indicated. 0.4 cc. of preincubated mixture assayed against cocoanut oil emulsion (30). Same incubation procedure used for lipase (steapsin).

in Table 5. The data in this table also indicate the complete dissimilarity of clearing factor and lipase, the latter being completely unaffected by either protamine or high ionic strength, both of which have marked inhibitory action on clearing factor.

Clearing factor is produced in high levels following the administration of anaphylactogenic agents into dogs. These agents presumably cause the release of both heparin and histamine from mast cells. Drs. Havel and Boyle (29) have recently shown that when the shock condition is prevented

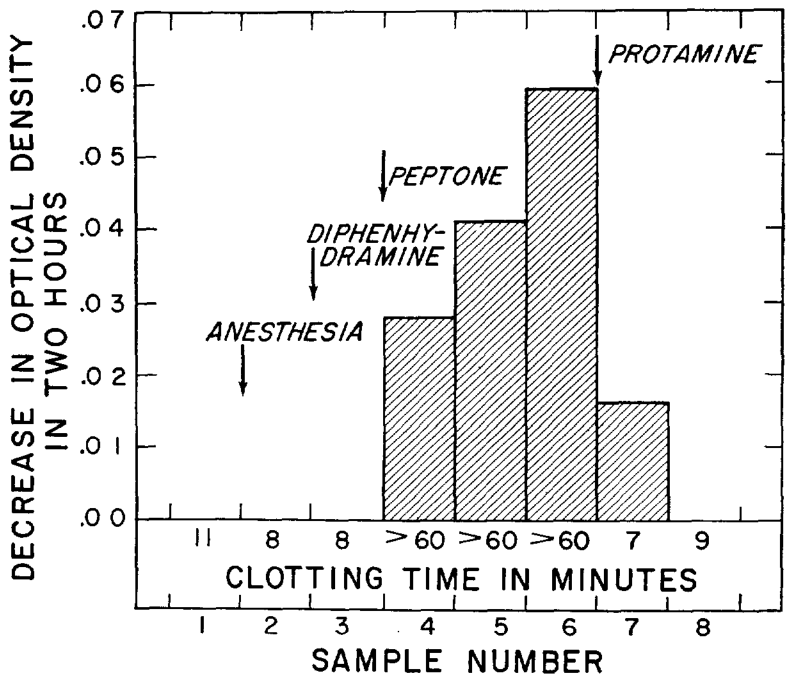

Figure 4. The production of clearing factor activity in the plasma of a dog following the production of shock with peptone. Anesthesia, anti-histaminic, and protamine were injected at the points indicated by the arrows. Plasma samples 1, 2, 3, and 8 contain no clearing factor activity.

by various antihistaminic agents, the plasma of these animals contains high levels of clearing factor activity, which disappears upon the subsequent injection of small amounts of protamine. The results of one such experiment are shown in Figure 4.

In general, therefore, although the presently available data is mainly indirect, it appears not unlikely that mechanisms for the normal production and utilization of clearing factor are present in normal animals.

Products of the clearing factor reaction. Evidence for the production of fatty acids and glycerol and for the probable intermediary accumulation of mono- and diglycerides during the action of clearing factor on low-density lipoproteins has been presented in the above sections. Although it is impossible, at present, to describe the exact fate of the protein moieties of these β lipoproteins, several well-defined physical changes have been studied ultracentrifugally and electrophoretically.

During the action of the clearing factor system on the low-density β lipoproteins of plasma (S_f 10 and above) these components appear to be degraded in the direction of β lipoproteins of higher density (15). Concomitantly, one observes the appearance of high density components with the ultracentrifugal properties of α_1 lipoproteins (17, 18). Such observations suggested originally (30) that the action of clearing factor might be due to " delipidation " with the production of an " α lipoprotein core." Alternatively, the process might involve the transfer of lipid molecules to protein components of plasma with densities originally too great to permit their visualization at the density level of the medium employed for ultracentrifugal analysis. Certain recent data obtained by Dr. Edwin Boyle have shown that the simple addition of long-chain fatty acids can cause the appearance in ultracentrifugal patterns of normal plasma of components with the centrifugal characteristics of both α and β lipoproteins. Although it is clear that these fatty acid-induced components are not identical with the normal α and β lipoproteins present in plasma which contain relatively well-defined levels of phospholipid, cholesterol, and protein, these results do indicate that the interpretation of the ultracentrifugal patterns of plasma should be made with some caution, particularly under circumstances in which fatty acids are being released, as occurs during the course of the clearing reaction. The physical changes induced by the *in vitro* addition of fatty acid to plasma or by the release of fatty acid following heparin injection, have been more thoroughly studied by electrophoretic methods (Dr. R. Gordon—unpublished data). Upon the administration of heparin to individuals whose plasma contains triglyceride-rich β lipoproteins, there occurs a rapid shift in the mobility of a portion of the β globulin peak. Such observations have also been made previously by other investigators, including Nikkilä (3), Rosenberg (31), and Lever et al. (32). In subsequent plasma samples withdrawn from the patient, this shift becomes more marked and the accelerated components may migrate with a mobility even greater than that of serum albumin as shown in Figure 5. With time, this shift is reversed and the electrophoretic pattern assumes its original appearance. Chemical analysis of pre-albumin components removed from the electrophoresis cell indicate a ratio of nitrogen to phosphorus of about 6 : 1, suggesting that the component which is binding the fatty acids released during the clearing are of the β_1 lipoprotein class. The reversion of the pattern to normal suggests the metabolic removal of fatty acid by tissues and raises the possibility that one or more components of the β globulin zone may act as transient carriers of fatty acid. *In vitro* support for this hypothesis is obtained from experiments in which pure S_f 3-8 β lipoprotein was titrated with sodium oleate, producing a marked shift in electrophoretic mobility. This shift was completely reversed by the addition of an approximately equal quantity

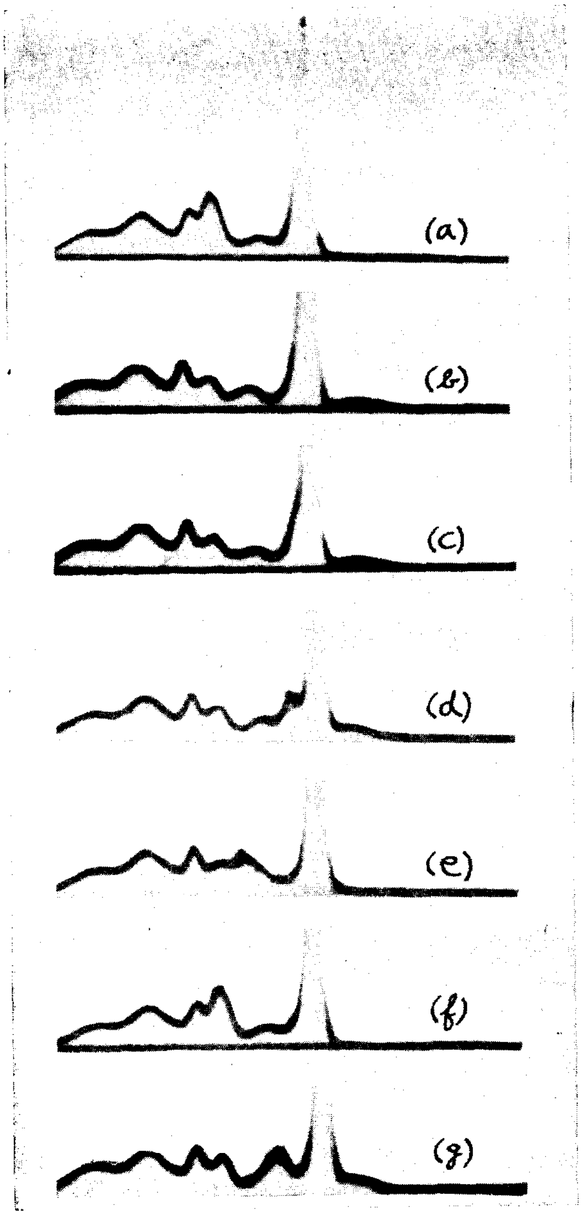

Figure 5. Electrophoretic patterns obtained on plasmas from a hypercholesterolemic patient before (a) and following (b, c, d, e, f) the administration of heparin, 0.75 mg kilo body weight. Pattern (g) is the pattern on plasma sample (a) following oleate addition *in vitro*. Phosphate buffer, pH 7.8, $\Gamma/2 = 0.16$.

of serum albumin, which competes more strongly for fatty acid.

In certain hyperlipemic patients with greater initial levels of low density lipoproteins, quite large shifts of mobility may be obtained by incubation, *in vitro*, of plasma samples withdrawn after heparin administration. Under these circumstances, the absence of metabolizing tissues accentuates the shift normally observed *in vivo*. These shifts in mobility occur very rapidly after heparin and the present results indicate the necessity for rapid chilling or salting of the plasma after withdrawal to permit the visualization of early changes. Heparin, in the amounts administered (.75 mg/kg) does not cause a visible change in the electrophoretic mobility of plasma protein components.

General Conclusions

Available evidence suggests that the accumulation of abnormal low density lipoproteins in plasma is the result of deficiencies in the mechanisms for normal degradation of these substances.

Our studies have indicated, in general, that the administration of heparin to individuals, both normal and metabolically disturbed, results in the production of essentially the same levels of clearing factor activity in their plasmas. These findings lead us to feel that, given adequate heparin, the mechanisms for producing and utilizing clearing factor may be generally unimpaired.

By analogy with the hormonal control of the blood sugar level through a homeostatic mechanism dependent on glucose concentration, one is led to consider the possibility that a similar homeostatic mechanism may apply in the case of lipids. The various investigations of the role of heparin in the metabolism of lipoproteins of plasma suggest that a major factor in such homeostasis might be the control of heparin secretion from mast cells, or its biosynthesis. It is felt that a thorough study of this biosynthetic process might lead to valuable advances in our understanding of the regulation of fat transport and mobilization in much the way that our knowledge of blood sugar level regulation has been enhanced by the study of glycogen synthesis and degradation under the action of hormonal agents.

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